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Acclimation to Drought Stress Enhances Oxidative Stress Tolerance in *Solanum lycopersicum* L. Fruits

Ramzi Murshed • Félicie Lopez-Lauri* • Cyril Keller • Fabien Monnet • Huguette Sallanon

Université d'Avignon et des Pays de Vaucluse, Laboratoire « Physiologie des Fruits et Légumes », F-84018 Avignon, France

Corresponding author: * **felicie.lauri@univ-avignon.fr**

ABSTRACT

A moderate water deficit stress (S1) applied 10 days after anthesis was found to induce acclimation to a subsequent more severe drought stress (S2) in *Solanum lycopersicum* L.. When plants were pretreated with S1, and then subjected to S2, fruit water status remained similar to controls. In contrast, S2 induced a decrease in both parameters of water status and diameter of fruits from non-acclimated (NA) plants. Following severe water stress, SOD, CAT and GR activities increased in NA fruits while APX and GR activities decreased in acclimated ones. On the other hand, membrane injury and H_2O_2 level increased in NA fruits submitted to S2. These results suggest that growing tomato fruits can acclimate to a moderate water deficit stress which moreover improves their ability to survive a more severe stress in the future with the development of less oxidative damage to fruits cells as indicated by MDA content.

Keywords: antioxidant systems, tomato fruit, water stress

Abbreviations: **APX**, ascorbate peroxidase; **AsA**, ascorbate; **CAT**, catalase; **DA**, drought acclimated; **DAA**, day after anthesis; **DHA**, dehydroascorbate; **DW**, dry weight; **FW**, fresh weight; **GR**, glutathione reductase; **GSH**, reduced glutathione; **GSSG**, oxidised glutathione; **MDA**, malonyldialdehyde; **NA**, non acclimated; **NBT**, nitro blue tetrazolium; **R**, rewatering; **ROS**, reactive oxygen species; **SOD**, superoxide dismutase; **TBA**, thiobarbituric acid; **TBARS**, thiobarbituric acid reactive substances

INTRODUCTION

Drought is one of the most important abiotic factors influencing the productivity of crops. Water deficit has detrimental effects on plant growth and development and induces several physiological, biochemical and molecular responses in plants. Many crops develop drought tolerance when subjected to gradual exposure to milder form of stress prior to a severe environmental stress (Khanna-Chopra and Selote 2007). The acquisition of drought tolerance involves the expression of specific genes that lead to stress-induced biochemical and physiological response to protect the plants from water deficit damage. Then, stress acclimation is associated with multiple mechanisms that include change in (a) gene expression (b) membrane composition (c) accumulation of osmoprotectants such as proline, sugars and (d) elevation of ABA and increased level of antioxidants (Smirnoff 1993). Many stresses, such as chilling, salinity and water deficit induce oxidative stress because of the inhibition of photosynthetic activity due to an unbalance between the quantities of light absorbed and used by photochemistry (Foyer and Noctor 2005). Changes in the photochemistry in the chloroplasts in the leaves of drought-stressed plants result in dissipation of excess light energy and may involve the generation of reactive oxygen species (ROS) ($O_2^{\bullet-}$, $^1O_2$, H_2O_2, OH^-), which are potentially dangerous (Apel and Hirt 2004). It may be argued that many stresses, because they induce stomatal closure and subsequently a decrease in photosynthesis, result in an increase in the production of ROS at different levels: the light collecting antennae ($^1O_2$) and the photosynthetic electron transport chain ($O_2^{\bullet-}$) in chloroplasts, and the peroxisomes (H_2O_2). Although the photosynthetic electron chain is potentially the most important source of ROS in plants, there is increasing evidence that ROS may also be produced by the respiratory electron transport chain in the mitochondria (Mittova *et al.* 2003).

These ROS cause oxidative damage to cellular components such as lipid peroxidation and consequently membrane damage, protein degradation, enzyme inactivation, pigment bleaching and disruption of DNA strands (Johnson *et al*. 2003; Halliwell 2006). Plants have developed two antioxidant defence systems, enzymatic and non enzymatic scavenging systems, to minimize the concentrations of ROS and protect plant cells from oxidative damages (Allen 1995). The key enzyme involved in the first steps of the ROS scavenging system is superoxide dismutase (SOD, EC 1.15.1.1) (Gomez *et al*. 2004). It catalyzes the dismutation of the superoxide anion ($O_2^{\bullet-}$) to oxygen and hydrogen peroxide (H_2O_2) (Fridovich 1986). Catalase (CAT, EC 1.11.1.6), guaiacol peroxidase or ascorbate peroxidase (APX, EC 1.11.1.11) convert hydrogen peroxide (H_2O_2) into non-toxic water (Asada 2006). APX enzyme is implicated in oxidation of AsA to monodehydroascorbate (MDHA), which can either dismutate back to ascorbate (AsA), or to dehydroascorbate (DHA). DHA is then rapidly reduced to AsA (Mano *et al*. 1997; Asada 2006). Glutathione reductase (GR, EC 1.6.4.2) is involved in the regeneration of AsA from MDHA via the NADPH-dependent reduction of oxidized GSSG to the reduced GSH (Noctor *et al*. 2002). The active ascorbate–glutathione cycle is responsible for efficient removal of excess ROS (Noctor and Foyer 1998; Asada 2006). In the non-enzymatic scavenging system, antioxidant compounds such as AsA, glutathione (GSH), carotenoids (lycopene, carotene), α-tocopherol, and many other phenolic compounds also play important roles (Foyer *et al*. 2005; Munné-Bosch 2005). AsA also participates in the regeneration of α-tocopherol, which traps alkylperoxyl radicals resulting from membrane lipid peroxidation and protects enzymes containing prosthetic transition ions.

Drought stress is associated with increased oxidative stress due to the enhanced production of ROS, particularly $O_2^{\bullet-}$ and H_2O_2 (Scandalios 1997). AsA can directly scavenge the H_2O_2 and thus protect the integrity of various membranes by preventing the conversion of H_2O_2 into highly po-

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tent OH radicals, which could induce lipid peroxidation leading to membrane damage (Navari-Izzo *et al*. 1994). APX in presence of high AsA content is involved in H_2O_2 scavenging and minimizes the subsequent oxidative conditions in the cell. Lesser oxidative damage in the tolerant wheat cultivar during osmotic stress has been attributed to higher AsA and GSH content, and induction of AsA–GSH cycle enzymes (Lascano *et al*. 2001).

The capacity to detoxify ROS has been related to stress tolerance in some earlier studies. Selote and Khanna-Chopra (2004) suggested that maintenance of favourable water relations in the drought-acclimated plants might have contributed towards the regulation of ROS generation (Selote *et al*. 2004). Khanna-Chopra and Selote (2007) showed clear difference in the participation of antioxidant defense system in the drought tolerance of wheat under field conditions when subjected to drought stress with and without acclimation treatment. In sunflower seedlings an induction of ascorbate–glutathione cycle enzymes during mild water stress minimized the oxidative damage during severe water stress conditions (Sgherri and Navari-Izzo 1995). Therefore, increased expression of antioxidant systems in plants may provide protection from ROS generated as a result of drought stress. The degree to which the activities of antioxidants enzymes and the amounts of antioxidants increase under drought stress is extremely variable among plant species (Zhang and Kirkham 1995), and even between cultivars of the same species (Bartoli *et al*. 1999).

Tomato, like many fruits and vegetables, is an important component of the diet in the Mediterranean zone, and it has been suggested that the consumption of tomatoes, which have high levels of antioxidants, may have a strong positive impact on public health by decreasing the risk of cardiovascular diseases (Weisburger 1999). Tomato plants are highly susceptible to drought stress notably during growth phase. Withholding water decreases leaf and fruit size, fruit number, the rate of photosynthesis and also fruit quality (Dodds *et al*. 1997). There is a considerable amount of literature concerning leaf acclimation to drought with respect to oxidative stress defense mechanisms, but little is known about such response mechanisms in fleshy fruits subjected to water deficit. In tomato plants, the changes in the antioxidants systems in response to different stresses have been investigated in detail in leaves. However, little is known about how drought changes the antioxidant systems in fruits. Pericarp of tomato fruits initially contains chloroplasts that are photosynthetically active, but they differentiate to non-photosynthetic chromoplasts during the ripening process. So, depending on the age of fruits, chloroplast photosynthesis or mitochondria respiration is a primary source of superoxide (Purvis *et al*. 1995).

The aim of the present work was to investigate the effect of drought-acclimation on tolerance to subsequent severe water deficit in tomato fruits. Not only does the study compare water status of fruits subjected to different treatments, it also addresses the issue of oxidative stress and acclimation of the antioxidant systems and major enzymes. We thus measured H_2O_2, membrane injury, SOD, CAT, APX, GR, AsA and DHA.

MATERIALS AND METHODS

Plant material and treatments

Tomato (*Solanum lycopersicon* L., cv. 'Raïssa', S&G® Seeds BV, The Netherlands) seeds were germinated in boxes filled with peat. When the first real leaf appeared, seedlings were transplanted into 4-L plastic containers containing a mixture of peat and vermiculite (2:1). Plants were transferred to a glasshouse and grown at a relative humidity of 60-70 % with day/night temperature of 28 ± 4/18 ± 4°C. The photoperiod was extended to 16 h d^{-1} at an average light intensity of 400 µmol m^{-2} s^{-1} with artificial light as required.

The plants were irrigated twice a week with a nutrient solution containing: 8 µM $MnCl_2$, 0.5 µM $CuSO_4.5H_2O$, 1.4 µM $ZnSO_4$, 46 µM H_3BO_3, 0.25 µM $Na_2MoO_4.2H_2O$, 4.1 mM KNO_3, 3.4 mM $Ca(NO_3)_2.4H_2O$, 0.9 mM K_2SO_4, 1 mM $MgSO_4.7H_2O$ and 1.5 mM KH_2PO_4 (Resh 1978; Sonnefeld and van der Wees 1984). The iron source was Fe-EDDHA chelate (0.6%). On the other days of the week, plants were irrigated with water.

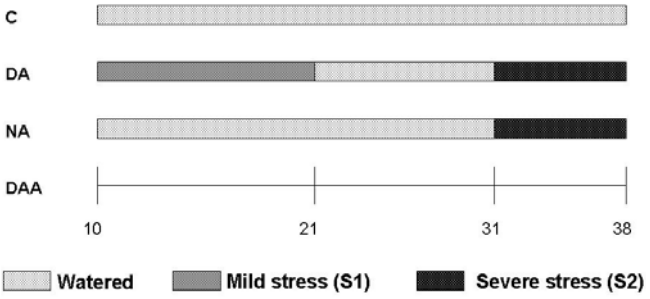

Fig. 1 Water stress treatments applied to tomato plants. C stands for control plants, DA for drought-acclimated plants. The latter were subjected to two periods of water stress, the first one of mild (S1), and the second one of severe (S2) intensity. R represents a period of rewatering. NA stands for non-acclimated plants. DAA: days after anthesis.

The stems of the plants were cut just above the third leaf above the second inflorescence and the inflorescences were thinned to three fruits per truss.

Plants were grouped into three sets and subjected to water stress treatments (**Fig. 1**). Water stress was started after fruit set, i. e. 10 days after anthesis (DAA). Control plants (C) were irrigated daily with an amount of water equal to the evapo-transpired water (approximately 1 L per day). Drought-acclimated plants (DA) were exposed to two successive stress periods, first a period of mild intensity (S1, between 10 and 21 DAA) followed by 10 days of rewatering (R), and finally a second period of severe intensity stress (S2; between 31 and 38 DAA).The non-acclimated plants (NA) were directly exposed to severe water stress conditions (S2). Tomato fruits remained green during all the time of experiments. In order to induce a moderate water stress (S1), DA plants were subjected to a water stress by gradually reducing the water supply: 85, 75, 65, 50, 35 and 20% of evapo-transpiration, at respectively 10, 12, 14, 16, 18, and 20 DAA. For the S2 treatment, a rapid reduction of water supply was applied: 75, 50, 25, and 10% of evapotranspiration at respectively 31, 33, 35 and 37 DAA.

The tomato fruits were harvested (at midday) at 21, 31, 38 DAA corresponding to growth stage. Pericarp was taken from fresh harvested fruits and immediately frozen in liquid nitrogen and ground. The powder was stored at -80°C.

Leaf and fruit water parameters

The predawn leaf water potential ($L\Psi_w$) of control and stressed plants was measured on the 3rd leaf after the second inflorescence using a pressure chamber according to the method of Scholander *et al*. (1965). Fruit water potential ($F\Psi_w$) was measured using the HR-33T Dew Point microvolt meter attached to C-52 chambers (Wescor Inc, Logan, Utah, USA). Fresh weight (FW) of leaves and fruits were determined just after harvesting. The dry weight (DW) was measured after that fresh material was dried at 70°C for 72 h. The water content was determined as: WC = [FW-DW]/FWx100.

Determination of H_2O_2 content

Hydrogen peroxide levels were determined according to Velikova *et al*. (2000). Frozen fruit powder (0.25 g) was homogenized in an ice bath with 1 mL 0.1% (w:v) TCA. The homogenate was centrifuged at 12,000 × *g* for 15 min at 4°C. Aliquots of 100 µL from each tube were placed in 96-well plates and 50 µL of 10 mM potassium phosphate buffer (pH 7.0) and 100 µL of 1 M KI were added in each well. Each plate also contained commercial H_2O_2 to generate a standard curve. Plate was briefly vortexes and the absorbance readings were taken at 390 nm in a micro-plate reader (PowerWave HT Microplate Spectrophotometer from BioTek). The content of H_2O_2 was given on a standard curve.

Determination of the malonyldialdehyde content

For the measurement of lipid peroxidation in fruits, the thiobarbituric acid (TBA) test, which determines malonyldialdehyde (MDA) as an end product of lipid peroxidation (Velikova *et al*. 2000), was used. Frozen fruit powder (0.25 g) was homogenized in 1 mL 0.1% (w/v) TCA solution. The homogenate was centrifuged at 12,000 × *g* for 15 min and 0.5 mL of the supernatant was added to 1 mL 0.5% (w/v) TBA in 20% TCA. The mixture was incubated in boiling water for 30 min, and the reaction stopped by placing the reaction tubes in an ice bath. Tubes were briefly vortexes and triplicate, 200 µL aliquots from each tube were placed in 96-well plates, and the absorbance of supernatant was read at 532 nm in a micro-plate reader. The value for non-specific absorption at 600 nm was subtracted. The amount of MDA–TBA complex (red pigment) was calculated from the extinction coefficient 155 $mM^{-1}.cm^{-1}$.

Antioxidant enzyme assays

All operations were performed at 4°C. For SOD and CAT activities, 1.5 g of frozen ground fruit material was homogenized in 5 mL of 100 mM phosphate buffer (pH 7.0) containing 1% (w/v) PVPP. For APX and GR activities, 1.5 g of frozen ground fruit material was homogenized in 5 mL of 50 mM Tris-HCl buffer (pH 7.0) containing 1 mM sodium ascorbate, 1 mM DTT, 1 mM EDTA, 1 mM reduced glutathione, 5 mM $MgCl_2$ and 1 % PVPP (w/v). The homogenate was centrifuged at 10000 × *g* for 15 min and the supernatant fraction was immediately assayed for enzyme activities and protein content.

Total SOD activity was determined by monitoring the inhibition of the reduction of *p*-nitro-blue tetrazolium chloride (NBT) (Ginnopolitis and Ries 1977). The reaction mixture containing 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 63 µM NBT, 50 µM riboflavin, 13 mM methionine and 50 µL extract was placed under a 22 W fluorescent lamp. The reaction was initiated by turning the light on and the reduction of NBT was followed by reading the absorbance at 560 nm for 10 min. Control reaction mixtures were prepared in the same way but incubated without illumination. One unit of SOD was defined as the amount of enzyme, which produced a 50% inhibition of NBT reduction.

Activity of CAT was determined by measuring the disappearance of H_2O_2 by decreased absorbance at 240 nm. The reaction mixture contained 50 mM phosphate buffer (pH 7.0), 10 mM H_2O_2 and 100 µL extract in a final volume of 2 mL (Aebi 1984).

APX activity was determined by following the decrease in absorbance at 290 nm due to the oxidation of ascorbic acid. The reaction mixture (2 mL) contained 50 mM phosphate buffer (pH 7.0), 1 mM ascorbic acid, 2.5 mM H_2O_2 and 100 µL enzyme extract (Knörzer *et al*. 1996).

GR activity was determined by following the rate of NADPH oxidation as measured by the decrease in the absorbance at 340 nm (Foyer and Halliwell 1976). The assay mixture (2 mL) contained: 50 mM Tris-HCl buffer (pH 7.5), 5 mM $MgCl_2$, 0.2 mM NADPH, 0.5 GSSG and 100 µL of the enzyme extract.

Total protein concentration in the enzyme extracts was determined by the method of Bradford (Bradford 1976), using bovine serum albumin as a standard.

All measurements were made at 25°C for each sample in triplicate.

Determination of ascorbate and dehydroascorbate concentrations

Total ascorbate (AsA plus DHA) and AsA concentrations were measured according to Kampfenkel *et al*. (1995), scaled down for micro-plates. A 0.5 g sample of frozen ground tomato fruit was homogenized in 1 mL of cold 6% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 16000 × *g* at 4°C for 15 min. The supernatants were used for total ascorbate and AsA determination.

For measurements of total ascorbate, 10 µL of extract were added to 10 µL of 10 mM DL-Dithiothreitol (DTT) and 20 µL of 0.2 mM phosphate buffer (pH 7.4). After incubation for 15 min at 42°C, 10 µL of 0.5% (w/v) *N*-ethylmaleimide (NEM) was added with incubation for 1 min at room temperature to remove excess DTT, followed by adding 150 µL of a reagent prepared just before use by mixing 50 µL of 10% TCA, 40 µL of 42% (v/v) of orthophosphoric acid (H_3PO_4), 40 µL of 4% (w/v) 2.2-bipyridyl dissolved in ethanol (70%) and 20 µL of 3% (w/v) ferric chloride. After incubation for 40 min at 42°C, the absorbance was measured at 525 nm in a micro-plate reader. For AsA determination, the same reaction was used but 0.2 M phosphate buffer (pH 7.4) was used in place of DTT and NEM. The amount of DHA was estimated from the difference of total ascorbate and AsA. Each plate also contained commercial L-Ascorbic acid to generate a standard curve.

Statistical analysis

The results are expressed as means with standard error (±SE). Statistical significance was determined by analysis of variance ($p<0.05$) using MINITAB Release 14 Statistical Software.

RESULTS

Drought tolerance

Moderate drought stress (S1), applied to provoke plant acclimation to drought, induced a significant decline of predawn $L\Psi_w$ and $F\Psi_w$, water content of fruits (FWC) and leaves (LWC) and fruit diameter (FD) as compared to control (C) ($P<0.05$) (**Tables 1, 2**). Rewatering (R) led to complete recovery of water parameters of leaves and fruits, as well as growth.

Exposure to severe water stress (S2) resulted in a decline in $L\Psi_w$ and LWC in drought-acclimated (DA) and non-acclimated (NA) plants as compared to control plants (C) ($P<0.05$). However, $L\Psi_w$ and LWC from acclimated plants (DA) were less affected by S2 (**Table 1**).

In contrast, fruit water parameters ($F\Psi_w$, FWC) and diameter of fruits (FD) from DA plants were not affected by S2 (**Table 2**). In addition, these parameters declined significantly in fruits from NA plants submitted to S2 ($P<0.05$), for example, the diameter of NA fruits was 30% lower than that of control and DA plants (**Table 2**).

Membrane injury and H_2O_2 content

Following mild stress treatment (S1) (**Table 3**), MDA and H_2O_2 increased in the fruits of plants subjected to water stress as compared to the control. During subsequent water stress of severe intensity, fruits from NA plants were characterized by higher concentrations of H_2O_2 and MDA. Rewatering (R) result in incomplete recovery of H_2O_2 and membrane injury levels. However, MDA and H_2O_2 levels of fruits from DA plants were similar to the levels observed in control fruits when S2 treatment was applied.

Antioxidant enzyme activities

In control plants, the activities of antioxidant enzyme increased with fruit growth (**Fig. 2**): SOD and APX activities continuously increased during fruit growth (**Fig. 2A, 2B**), while GR and CAT activities increased at 21 and 31 DAA and remained stable between 31 and 38 DAA (**Fig. 2C, 2D**).

During mild water stress, significant enhancement in the activities of antioxidant enzymes SOD, APX and GR was observed in acclimated tomato fruits (**Fig. 2**). SOD activity increased 2.5-fold in acclimated fruits (**Fig. 2A**). In contrast, CAT activity was not affected by mild stress (**Fig. 2D**).

Recovery treatment (R) did not produce significant effects on enzyme activities; SOD and APX activities remained higher than in the control; moreover GR activity was similar to the control (**Fig. 2**).

S2 resulted in a significant decrease of APX activities in the acclimated and NA fruits ($P<0.05$), which exhibited a more significant decline in APX activity compared to acclimated ones ($P<0.05$). Moreover, an increase of GR activity in NA fruits as compared to acclimated fruits was noted

Table 1 Predawn leaf water potential ($L\Psi_w$; MPa) and water content of leaves (LWC; %) of control plants (C), drought-acclimated plants (DA) and non-acclimated plants (NA) at the end of the mild water stress period (S1), the rewatering period (R) and the severe water stress period (S2). Values are means (± S.E.) of measurements from five plants for each treatment (n = 5). *: indicates significant differences from the control value determined by analysis of variance (ANOVA) at 5% level.

	S1		R		S2		
	C	DA	C	DA	C	DA	NA
$L\Psi_w$	- 0.33 ± 0.03	- 0.66 ± 0.04 *	- 0.37 ± 0.02	- 0.44 ± 0.01	- 0.30 ± 0.03	- 1.20 ± 0.09 *	-1.50 ± 0.07 *
LWC	85.9 ± 0.8	83.7 ± 0.9*	86.9 ± 0.3	87.0 ± 0.2	87.0 ± 0.2	82.8 ± 0.3 *	80.9 ± 0.4 *

Table 2 Fruit water potential ($F\Psi_w$; MPa), water content of fruits (FWC; %) and fruit diameter (FD; cm) of control plants (C), drought-acclimated plants (DA) and non-acclimated plants (NA) at the end of the mild water stress period (S1), the rewatering period (R) and the severe water stress period (S2). Values are means (± S.E.) of measurements from five plants for each treatment (n = 5). *: indicates significant differences from the control value determined by analysis of variance (ANOVA) at 5% level.

	S1		R		S2		
	C	DA	C	DA	C	DA	NA
$F\Psi_w$	- 0.61 ± 0.02	- 1.11 ± 0.27 *	- 0.50 ± 0.04	- 0.50 ± 0.09	- 0.71 ± 0.07	- 0.71 ± 0.08	- 0.85 ± 0.03 *
FWC	94.2 ± 0.2	91.1 ± 0.3 *	94.0 ± 0.3	93.7 ± 0.8	94.4 ± 0.6	94.1 ± 1.0	91.1 ± 0.6 *
FD	1.24 ± 0.02	0.90 ± 0.04 *	4.40 ± 0.18	3.54 ± 0.20	13.15 ± 0.34	12.33 ± 0.38	8.75 ± 0.24 *

Table 3 Change of H_2O_2 ($\mu g.g^{-1}$ FW) and MDA ($\mu mol.g^{-1}$ FW) contents of control plants (C), drought-acclimated plants (DA) and non-acclimated plants (NA) at the end of the mild water stress period (S1), the rewatering period (R) and the severe water stress period (S2). Values are means (± S.E.) of measurements from five plants for each treatment (n = 5). *: indicates significant differences from the control value determined by analysis of variance (ANOVA) at 5% level.

	S1		R		S2		
	C	DA	C	DA	C	DA	NA
H_2O_2	3.2 ± 0.25	6.4 ± 0.4 *	4.2 ± 0.3	5.4 ± 0.2 *	5.2 ± 0.25	5.3 ± 0.3	8.7 ± 0.4 *
MDA	32 ± 1.8	54.3 ± 2.1 *	37 ± 1.5	41.5 ± 1.75*	43.5 ± 1.6	41.7 ± 1.9	68.8 ± 2.1 *

Table 4 Total ascorbate, Ascorbate (AsA) and dehydroascorbate (DHA) concentrations ($\mu moles\ g^{-1}$ DW) and ascorbate redox state (AsA/Total) in fruits of control plants (C), drought-acclimated plants (DA) and non-acclimated plants (NA) at the end of the mild water stress period (S1), the rewatering period (R) and the severe water stress period (S2). Values are means (± S.E.) of measurements from five plants for each treatment (n = 5). *: indicates significant differences from the control value determined by analysis of variance (ANOVA) at 5% level.

	S1		R		S2		
	C	DA	C	DA	C	DA	NA
Total AsA	19.46 ± 0.84	16.92 ± 0.95	20.77 ± 2.46	16.64 ± 3.03	29.96 ± 1.64	28.88 ± 0.83	34.41 ± 3.02 *
AsA	14.07 ± 0.44	13.30 ± 1.12	18.33 ± 2.25	14.73 ± 3.14	24.94 ± 1.38	24.25 ± 0.87	33.94 ± 2.99 *
DHA	5.39 ± 0.72	3.62 ± 0.23	2.44 ± 0.50	1.91 ± 0.35	5.02 ± 1.33	4.64 ± 0.75	0.47 ± 0.10 *
AsA redox state	0.73 ± 0.03	0.78 ± 0.02	0.88 ± 0.03	0.87 ± 0.04	0.84 ± 0.04	0.84 ± 0.03	0.99 ± 0.01 *

(**Fig. 2C**). In contrast, the SOD and CAT activities were not notably affected in acclimated plants while they increased in NA fruits.

Non-enzymatic antioxidants

Total ascorbate and AsA concentrations increased in tomato fruits during development and growth (**Table 4**). The amounts of total AsA, AsA and DHA in fruits were not significantly modified by either the mild water stress (S1), the rewatering period (R) or severe stress (S2) in drought-acclimated plants (DA). Nevertheless, fruits of Na plants subjected to S2, exhibited an increase in AsA of 27% as compared to the control, while, at the same time, the level of DHA significantly declined in these fruits ($P<0.05$). The AsA redox state decreased only in NA fruits subjected to S2 (**Table 4**).

DISCUSSION

Many researches have shown that stress tolerance might be acquired by exposure to a non lethal and progressive stress treatment before the application of a more severe constraint (Khanna-Chopra and Selote 2007). The growth phase is generally believed to be the most critical stage, i.e. the most sensitive to drought stress. Therefore, all experiments were made during cell growth (between 10 and 38 DAA) and not during maturation of fruits.

The development of fruits requires a major investment of carbon and water. Photosynthetic contributions by young green fruits to their daily carbon gains represent less than 10%, and over 90% is supplied via the phloem from the rest of the tomato plant. Because the translocation of photoassimilates from leaves to developing fruits requires considerable amounts of water (Ho *et al.* 1987; Nobel and de la Barrera 2000), drought-stressed fruits do not grow. Our results confirm that the decrease of water availability reduced fruit growth and that maintenance of favourable plant water relations is vital for the development of drought resistance in plants (Passioura 2002). In our study, we showed that moderate as well as severe water stress reduced tomato fruit size in the plants stressed at 10 DAA or 31 DAA as previously described by Mingo *et al*. (Mingo *et al*. 2003). Moreover, the restriction in water supply was clearly accompanied by a significant decline of water content and water potential of leaves and fruit pericarp (**Tables 1, 2**). During the rewatering phase, the water content and water potential increased rapidly, as well as fruit size, to reach the levels observed in the control fruits. Tomato fruits harvested from drought acclimated plants (DA) were less affected by the severe stress (S2) than non-acclimated plants (NA), although the leaves of DA and NA plants suffered from water loss and from decreased water potential (**Tables 1, 2**). Compared to the control, the fruits from NA plants were smaller and the water content was lower (**Table 2**) whereas fruit size, water potential and water content of DA fruits remained similar to those of controls. Thus, fruit pericarp displays a better ability to acclimate to water deficit than do the leaves. Data concerning the relationships between fruit growth and fruit water potential, would suggest that cell expansion was regulated by water potential and turgor in the pericarp as proposed by Davies *et al*. (2000). However non-hydraulic regulation of growth has been shown in tomato fruit (Mingo *et al*. 2003).

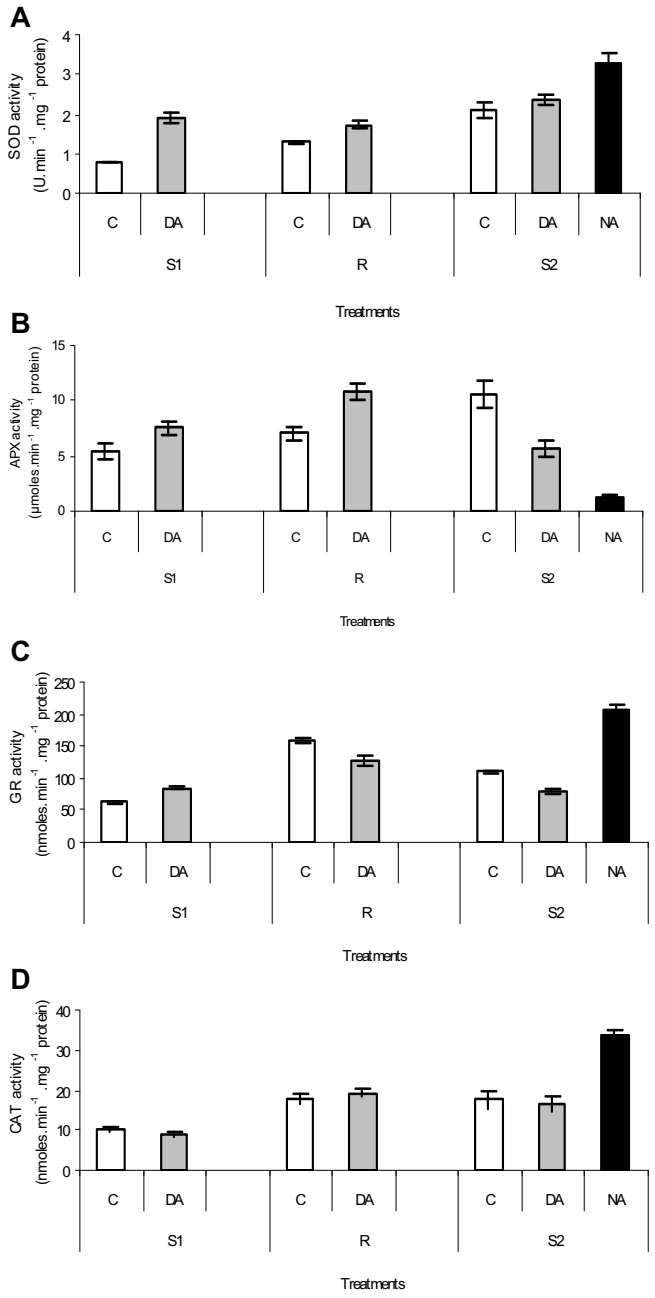

Fig. 2 Activity of superoxide dismutase (SOD) (A), ascorbate peroxidase (APX) (B), glutathione reductase (GR) (C) and catalase (CAT) (D) in fruits of control plants (C), drought-acclimated plants (DA) and non-acclimated plants (NA) at the end of the mild water stress period (S1), the rewatering period (R) and the severe water stress period (S2). Values are means (± S.E.) of measurements from five plants for each treatment (n = 5).

The drought acclimation of DA fruits was also reflected by the non generation of membrane injury, estimated by the TBARS test, in response to the severe stress (S2). In fact, MDA level in DA fruits remained similar to the control level when plants were subjected to S2 treatment while drought stress caused a significant increase in MDA levels in non acclimated plants. The lesser membrane damage in acclimated plants might be due to a lower production of ROS and particularly of H_2O_2 (**Table 3**). Khanna-Chopra and Selote (2007) reported that drought acclimation treatments by exposure to mild water stress enabled wheat seedlings to limit ROS accumulation during subsequent exposure to severe water stress. Recent reports suggest that H_2O_2 in low concentrations can serve as a second messenger in signal transduction, leading to acclimation (Foyer *et al*. 1997; Dat *et al*. 2000; Desikan *et al*. 2001). Pre-treatment of wheat seeds with increased H_2O_2 levels, alleviated the deleterious effect of salinity on seedling growth by a minimal change of growth parameters, such as leaf area, of water relation, such as water potential and turgor, and membrane permeability (Wahid *et al*. 2007). Following these authors, it might be hypothesized that H_2O_2 produced in the pericarp during the moderate drought pre-treatment (S1) and during rewatering phase is involved in drought tolerance of DA fruits, specifically by enhanced protection of membrane integrity and stability.

On other hand, hydrogen peroxide is a strong oxidizing agent and it is vital for plants to adjust the enzymatic and non-enzymatic antioxidant systems to control the amounts of ROS to avoid oxidative stress (Allen 1995). The regulation of the activities of SOD and ascorbate-glutathione cycle enzymes is a rapid and efficient response to limit the excess of ROS generated by drought.

Superoxide dismutase converts $O_2^{\bullet-}$ to H_2O_2 another toxic ROS. An increase in SOD activity was observed in the pericarp at the end of acclimation stress (S1) at 21 DAA. Again, increased SOD activity was observed in NA fruits subjected to S2. This observation suggests that the moderate, like the severe stress induced ROS accumulation in tomato fruits as observed by Rahman *et al*. (2000). In fruits of DA plants, the SOD activity did not increase as a consequence of the S2 treatment. Since the increase in SOD activity is circumstantial evidence for enhanced production of ROS in tomato fruit, it may be hypothesized that no ROS were produced in response to stress in fruits from drought-adapted tomato plants (**Table 3**).

The activity of ascorbate peroxidase, which is capable to scavenge H_2O_2 produced by SOD, and GR, involved in ascorbate-glutathione cycle, were significantly increased during the drought acclimation treatment (S1). On the other hand, the activities of APX, as well as SOD activity remained significantly enhanced relative to the control during recovery from moderate drought stress (**Fig. 2**). Moreover APX activity was higher than in fruits subjected to the water deficit pre-treatment. Other studies on oxidative stress have shown that SOD, APX and GR and their transcripts were higher during recovery than when subjected to periods of drought, as observed, for example, in pea (Mittler and Zilinskas 1994) or cotton (Ratnayaka *et al*. 2003) leaves. Thus, the increased enzyme activities in the drought conditioned seedlings may have contributed to the alleviation of the water deficit injury of tomato by scavenging efficiently ROS in excess, resulting in a complete recovery of water content and $F\Psi_w$ of fruits was reached. When the water stress was applied at the end of growth period (31 DAA), APX activity declined in fruits of DA plants compared to control. Moreover, the decrease of APX activity was greater in fruits of NA plants in which SOD activity increased, suggesting that there was a production of H_2O_2. Catalase activity scavenges H_2O_2 and an increase in its activity is also related to drought response. Indeed, CAT activity only increased significantly in NA plants subjected to severe stress (**Fig. 2D**), which is consistent with the necessity to eliminate H_2O_2 produced by enhanced SOD activity (**Fig. 2A**). As attempted, no significant CAT induction was observed in DA plants in response to S2. Consequently, failure in the antioxidant ascorbate-glutathione defense system at the end of growth period, as suggested by decreased APX (**Fig. 2**), may result in induction of peroxisomal catalase when needed. Rizhsky *et al*. (2002) showed that APX deficiency result in induction of CAT. Our results suggest that the response of the APX/GR system to drought stress varies according to the development stage (beginning or middle of the growth period) and the intensity of the stress (mild (S1) or high (S2)).

We observed in the fruits of DA plants that the APX-GR enzymatic system was down regulated during the period of severe stress (S2), and that this downregulation was not accompanied by any induction of SOD activity. Moreover, growth and water parameters, as well as the level of membrane injury of fruits from DA plants subjected to S2, seem

to be similar to control fruits, which suggests that DA fruits were not affected by stress. Therefore, it may be hypothesized that decreased APX and GR activities in response to the S2 treatment were not related to water status in DA fruits, while probably resulting from a signal originating from the leaves (**Table 2**).

GR also plays a role in oxidative stress by converting oxidized glutathione (GSSG) into reduced glutathione (GSH). The elevated level of GR activity observed in NA fruits could increase the GSH/GSSG ratio, which is required for AsA regeneration. AsA participates in redox regulation in different cell compartments to protect plants from oxidative damage caused by drought (Noctor and Foyer 1998). In our study, compared with the control, the concentration of AsA only increased in fruits of NA plants during S2 (**Table 3**). On the other hand, DHA concentration remained unchanged, with the exception of a significant decrease in fruits from NA plants (**Table 3**). These results suggest that in fruits at 38 DAA from NA plants, drought stress induced an antioxidant response of tomato fruits which could be mediated by CAT and GR but not by APX and SOD depending of age of pericarp cells. This response leads to increase of AsA accumulation via probable induction of dehydroascorbate reductase (DHAR) and GR.

Another important result of the present study is the increase of antioxidant enzyme activities, ASA and DHA, during the growth of fruits. To date, only a few studies have addressed this point because the authors have focused on ripening fruits (Rabinowitch *et al*. 1982; Rogiers *et al*. 1998; Jiménez *et al*. 2002; Mondal *et al*. 2004). Our data showed an increase in SOD, APX, GR and CAT activities and in ascorbate concentration during fruit development. There are conflicting data concerning changes in antioxidant enzyme activities during fruit growth of tomato resulting perhaps from the cultivar used for experiments. Our observation were comparable to those of Mondal *et al.* (2004), who showed that SOD, APX, GR and CAT activities increased between immature green and mature green stage in two cultivars of *S. lycopersicon* L (set-7 and ARTH-3). However, in Ailsa Craig tomatoes, these enzymatic activities declined between the same stages (Jiménez *et al*. 2002).

In conclusion, submitting fruits to a mild drought during the first period of fruit growth provides a great advantage in that sense that it prepares them to cope with a more severe upcoming stress. The data presented suggest than acclimation of tomato plants by a moderate water deficit during the early fruit growth phase could prevent arrest of growth and also minimize fruit dehydratation, while preventing the accumulation of ROS and membrane lipid peroxidation during a future drought phase.

In addition, the work presented has provided new insights into the physiological role of some of the enzymes of the antioxidant systems during fruit growth under stress conditions. Drought stress induces SOD and APX in young tomato fruits while SOD, CAT and GR were induced, while APX activity decreased at the end of growth period. This observation also suggests that the mechanism of regulation of APX and GR are different.

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